IMMUNOLOGICAL, BIOCHEMICAL AND CHEMICAL FEATURES OF CIGUATOXINS: IMPLICATIONS FOR THE DETECTION OF CIGUATERIC FISH

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A major advance in the management of ciguatera will come with the development of a validated, cost-effective assay that detects ciguatoxins contaminating fish. Progress towards such a goal is summarised and the implications for detection of the toxins involved in ciguatera are discussed. Ciguatera results predominantly from CTX-1 which is present at >0.1ppb (10^{-10} mole/kg) in the flesh of carnivorous fish. Other toxins in ciguateric fish are likely to have no more than a minor role in ciguatera. Consequently, CTX-1 should be the principal target of any assay for ciguateric fish. However, significant levels of the less potent ciguatoxins, particularly ciguatoxin-2 and -3, may also accumulate in fish and such toxins could potentially interfere with the response of an assay. Ciguatoxins-1, -2 and -3 have an affinity for voltage-dependant sodium channels (ED_{50} = 0.2 –0.9nM) that is proportional to their i.p. LD_{50}s in mice. Assays (biosensors) that measure this binding or perhaps the ciguatoxin induced sodium channel opening may provide a sensitive assay for ciguatoxins with a response proportional to toxin potency. Ciguatoxins also bind to a range of other proteins, which may interfere with the response of some assays. Alternatively, such interactions may be utilised in the development of novel sandwhich-type assays. Ciguatoxins-1, -2 and -3 do not possess a useful chromophore for selective spectroscopic detection; however, each possesses a relatively reactive primary hydroxyl through which a label could be attached (after appropriate clean-up) prior to detection. Detectors (e.g. fluorescence or mass spectrometry) coupled to optimised HPLC may provide the required sensitivity for analytical detection of derivatised ciguatoxins in crude extracts of fish. Such assays could replace the mouse bioassay for the validation of responses obtained by more rapid screening assays.

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Ciguatera is a disease with a wide array of gastrointestinal and neurological symptoms. It stems mostly from the effects of ciguatoxin-1 (Fig.1), the most potent of the ciguatoxins (Murata et al.,1990; Lewis et al.,1991; Lewis & Sellin,1992). The disease can be debilitating and slowly resolving but is seldom fatal. In many Western countries outbreaks of ciguatera often attract media attention with a consequent negative impact on the marketing of seafood and victims of ciguatera may seek compensation through the courts. A cost-effective means of detecting ciguateric fish prior to consumption remains one of the few management options that can directly reduce the adverse impacts of ciguatera. Antibody-based assays appear to hold most promise since they are able to detect, under favourable circumstances, targeted compounds to 10^{-12} M and can be developed as cost-effective screens (Gazzaz et al.,1992). This paper reviews antibody-based assays for the identification of ciguateric fish and discusses immunological, biochemical and chemical features of the ciguatoxins relevant to their detection.

DEVELOPMENT OF ANTIBODY-BASED ASSAYS FOR CIGUATERIC FISH

The potential of an antibody-based screening assay for detecting ciguatoxin in fish flesh was first indicated by Hokama et al. (1977). Hokama has since led efforts to develop a rapid screen for ciguateric fish (Hokama,1991). The original radioimmunoassay screened 88% of moray eel and 38% of other fish as toxic (>3.5 x 10^5cpm/g; Kimura et al.,1982) despite these fish rating as non-toxic by the mongoose assay. Despite this high false positive result, all fish rating toxic by the mongoose rated as toxic by the antibody assay, indicating the potential for this approach to detect ciguateric fish. This assay was subsequently employed to screen 5,529 *Seriola dumerili* captured in Hawaiian waters (Kimura et al.,1982). This study found 15% of *S. dumerili* tested positive, with the remaining fish, including those >9kg which normally are not marketed owing to their perceived higher risk of ciguatera, being consumed without incident. The quantity of additional *S. dumerili* entering the market in-

creased 68% as the direct result of the study. However, Kimura *et al.* (1982) found that 7% of fish (3 of 42) clinically implicated in ciguatera tested negative by the radioimmunoassay.

In 1984 the radioimmunoassay for ciguateric fish was replaced by a simpler enzyme immunoassay that in binding inhibition assays was sensitive to as little as 5pg of free ciguatoxin (Hokama et al., 1983,1984). The cross-reactivity of this assay with other polyether toxins was also documented. An enzyme labelled polyclonal antibody was subsequently used to develop a further simplified 'stick-test' that rapidly distinguished toxic from non-toxic flesh samples (Hokama, 1985); however six tests per fish appeared necessary for accurate determination of ciguateric fish that tested close to the borderline level. With the stick-test the rejection rate for *S. dumerili* was only 11% and *S. dumerili* testing non-toxic were consumed without incident. This assay responded directly to >1.0ng of pure ciguatoxin (Hokama,1985).

These early studies all employed a polyclonal antibody raised to ciguatoxin in sheep with the disadvantage that for long-term antibody production a continual supply of antigen is required for booster injections. Monoclonal antibodies, on the other hand, can provide a continuous supply of a selected antibody. Hokama et al. (1985,1989b) reported production of monoclonal antibodies to a related polyether toxin okadaic acid as well as to ciguatoxin (likely CTX-1). Using a monoclonal IgG to ciguatoxin in a stick enzyme immunoassay, Hokama et al. (1989a) found that 98% of fish implicated in ciguatera (50 of 51) tested positive, while 55% of a random mix of fish, 55% of *Ctenochaetus striatus* and 44% of *S. dumerili* tested positive. A further simplified solid-phase immunobead assay for detection of ciguateric fish (Hokama,1990) appeared more sensitive than previous stick tests.

The monoclonal antibody to ciguatoxin used in these studies has been assessed for cross-reactivity to other polyether toxins (Hokama *et al.*, 1989b,1992). The assay employing the antibody raised to ciguatoxin detected similar concentrations of pure ciguatoxin, okadaic and a synthesised fragment of okadaic acid (EC_{50} = ~ 0.5 ng of toxin per ml methanol). The cross-reactivity of an antibody for ciguatoxin and okadaic acid is somewhat unexpected, given recent molecular modelling studies which show that in solution the structure of okadaic acid is quite different from that of ciguatoxin (Norte et al.,1991; Lewis and Ramsdale, unpublished observations). It is also intriguing that a monoclonal antibody obtained specifically to okadaic acid was less sensitive at detecting okadaic acid (EC_{50} ~15ng/ml) than the ciguatoxin antibody using the same assay format. Somewhat different cross-reactivity was reported in an earlier study (Hokama et al.,1989b). Importantly, addition of pure ciguatoxin (EC_{50} ~1 ng/ml) and okadaic acid (EC_{50} ~3ng/ml) to a ciguatoxin antibody inhibited the subsequent binding of this antibody to stickscoated with an extract from a fish implicated in ciguatera. This result suggests these polyether toxins compete at a specific, saturable site on the IgG. Since ciguatoxin alone can bind to correction fluid coated sticks (the Hokama poke stick method is based on the ability of sticks dipped into an alchohol-based typist's correction fluid to extract and immobilise CTX prior to detection of this immobilised CTX with labelled antibody) and subsequently bind antibody but the preformed antibody-CTX complex is no longer able to bind to such sticks through the CTX link, it is possible that CTX binding sites on the coated sticks are also saturable.

Development of a commercial screening assay for ciguateric fish is being undertaken by Hawaii Chemtec International who purchased development rights for the Hokama stick test from the University of Hawaii. Recent findings are given in other articles in this memoir.

IMMUNOLOGICAL FEATURES

An antibody response is elicited in an animal following injection of an immunogenic antigen. The ciguatoxins ($m/z = 1,094–1,110$) are relatively small haptens that are likely to have only low immunogenicity. The absence of any protection in people repeatedly exposed to ciguatoxins through their diet supports this suggestion. A hapten will become immunogenic when covalently conjugated to a carrier protein possessing high immunogenicity (Erlanger,1980). However, non-covalent conjugation and/or Fruends' complete adjuvant are unable to significantly enhance the immunogenicity of native haptens (Layton et al.,1987; Gazzaz et al.,1992). One draw-back with having to use a hapten conjugated to a carrier protein is that the antibodies obtained often do not have high specificity for the unconjugated (native) form of the hapten.

Ciguatoxins possess a relatively reactive primary hydroxyl (Murata et al.,1990; Lewis et al.,1991; Lewis et al.,1993) which can be reacted with succinic anhydride to yield a hemisuccinate.

The hemisuccinate so formed has an available carboxyl group through which ciguatoxin can now be linked to a carrier protein (e.g. bovine serum albumin, keyhole limpet haemocyanin, ovalbumin) using a water soluble carbodiimide cross-linking reagent. Ciguatoxin cross-linked to a carrier protein in this way is expected to have considerably enhanced immunogenicity compared with the native ciguatoxin. Such a complex may additionally have reduced potency, an important consideration for *in vivo* immunisation. The availability of only one easily accessible site on ciguatoxin for conjugation to a protein limits (Mandal & Latif, 1988) the possibilities for producing a range of antibodies possessing differing selectivities for the various ciguatoxin analogues. To-date antibodies to ciguatoxin have not been obtained using an immunogen covalently attached to a carrier protein. However, the use of such an immunogen is presently under consideration in a number of laboratories. Attempts in our laboratory to produce a hemisuccinate of ciguatoxin-1 have met with little success, despite the use of succinic anhydride in dried pyridine at 80°C (Baden et al.,1984), a method that we successfully used to produce a hemisuccinate of brevetoxin-3 (Lewis unpubl. data).

To obtain a maximal *in vivo* immune response with small quantities of immunogen, the immunogen should be emulsified in Fruends' complete adjuvant prior to injection (Vaitukaitis, 1981; Smith et al.,1992). Blood from suitably immunised animals is then collected and assessed for selective antibody titre against the compound of interest. For monoclonal antibody production, the spleen cells of immunised mice are fused with a murine myoloma cell line, with hybridoma(s) secreting ciguatoxin-specific immunoglobulins subsequently isolated using an appropriate screen. This approach follows well described procedures (Galfrè & Milstein,1981; Goding,1986; Peters & Baumgarten,1992). Alternatively, monoclonal antibodies can be obtained through *in vitro* immunisation procedures (Brazeau et al., 1982; Van Ness et al.,1984; Buchman et al.,1985; Borrebaeck,1986; Brams et al.,1987; James & Bell,1987; Borrebaeck & Glad,1989). This approach allows the production of high specificity antibodies (including human antibodies) with small quantities of immunogen and compared with *in vivo* immunisation is less susceptible to immunogen toxicity. Modifications to standard *in vivo* immunisation protocols can also reduce the quantity of immunogen required (Vaitukaitis,1981; Forest & Ross,1993). Such *in vitro* and *in vivo* approaches may be useful for production of antibodies to ciguatoxin.

To allow detection of potentially useful antibodies, a screen must be developed that is selective only for those antibodies that combine with high affinity to the compound being targeted. Such a screen may utilise a hapten-protein conjugate that is attached, through non-specific interactions, to the plastic surface of microtitre plates. To avoid potential problems of cross-reactivity to carrier protein or to epitopes extending beyond the hapten itself, a protein *and* cross-linking reagent should be used in screening that is different from that used in the preparation of the immunogen. Additionally, any method employed to couple ciguatoxin to a carrier protein should not alter the structure of the targeted compound, otherwise antibodies may be produced that will not recognise the native compound. This is equally important for compounds labelled for use as the competitor in competitive antibody binding assays. The careful choice of blockers (Tween 20, fetal calf serum, albumins etc) must be made to ensure that the response is specific for the compound of interest (e.g. ciguatoxin). One pitfall is the ability of certain clones to express antibody which binds promiscuously to plastic (Conger *et al.*,1988). We have noted that exposing plastic microtitre plates to methanol-water (1:1) increases the plate's affinity for IgG, an effect that could not be blocked by traditional blockers and gave rise to a number of false postive results.

Ciguatoxin recognising antibodies obtained in an appropriate manner, and with confirmation that they are specific for only the CTX-class of polyethers, could then be used in the detection of ciguateric fish. Screening assays employing antibodies that have both high affinity and selectivity for an epitope on ciguatoxin-1 are essential if routine detection of the low levels of ciguatoxins (10^{-10} to 5 x 10^{-9}g CTX-1/g) contaminating the flesh of ciguateric fish is to be achieved. Cross- reactivity to other ciguatoxin congeners may also be acceptable. Ideally the chosen antibodies should have an affinity that is directly proportional to the oral potency (to humans) of the contaminating toxins and should not cross-react with compounds normally present in non-toxic fish. The high cross-reactivity of ciguatoxin antibodies to less potent and structurally dissimilar polyether toxins such as okadaic acid (Hokama et al.,1989b,1992) may in part explain the high number of false positive results obtained with assays employing such antibodies. Recent studies using steroid-antibody

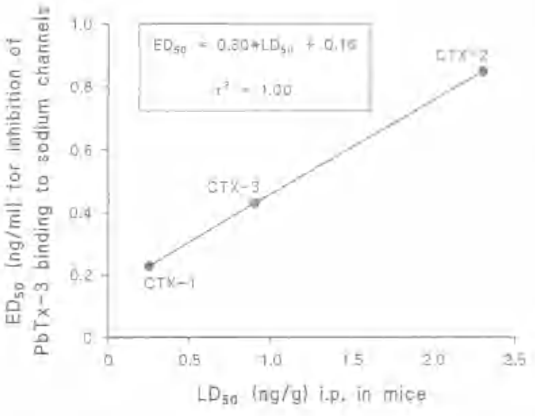


FIG. 1. Relationship between sodium channel binding affinity and mouse lethality for three ciguatoxins.

interactions as a model, have revealed that antibodies recognising apolar and functionally inert molecules (such as ciguatoxin) can have a high afinity binding site but that this site apparently can't be engineered with high specificity to avoid its cross-reactivity with related ligands (Arevalo et al.,1993). In contrast to antibodies that recognise ciguatoxin, and somewhat surprisingly given the previous statement, antibodies raised to brevetoxin and okadaic acid have been found to possess low cross-reactivity with other polyethers including CTX-1, -2, and -3 (Levine et al.,1988; Lewis et al.,1991; Poli et al.,1992) and it remains to be confirmed if it is possible to obtain brevetoxin-antibodies that cross-react with ciguatoxin. Non-selective bindings of ciguatoxin to IgG and non-selective binding of IgG to fish tissue (Parc et al.,1979; Chanteau et al.,1981; Emerson et al.,1983) may present additional obstacles to the development of a successful screening assay for ciguateric fish. The limited experience of other laboratories with several prototype assays utilising polyclonal (Berger & Berger,1979) and monoclonal antibodies (Lewis, unpubl. data) developed by Hokama have at times given less than satisfactory results that are difficult to explain.

BIOCHEMICAL FEATURES

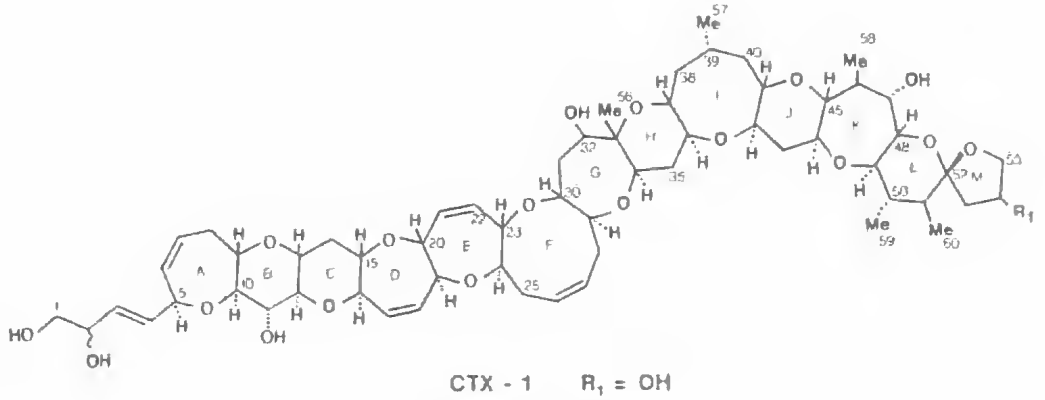
Ciguatoxin binding to sodium channels

Ciguatoxins are characterised by high affinity binding (ED_{50} = 0.23–0.85ng/ml) for voltage sensitive sodium channels (Lewis et al.,1991). The binding affinity of each ciguatoxin for the sodium channel (ED_{50}) is proportional to its i.p. LD_{50} in mice (Lewis et al.,1991) indicating that the lethal effect of the ciguatoxins likely stem from their action on sodium channels. Interestingly, this relationship is found to be linear ($ED_{50} = 0.30 \times LD_{50} + 0.16$) for CTX-1, -2 and -3 (Fig. 1). That this relationship has a slope of 0.3 indiates that binding affinity is not directly proportional to lethality and additional factor(s), presumably pharmacokinetic (Lewis et al.,1991) attenuate the lethality of the ciguatoxins with lower binding affinity. Since these ED_{50}s are a measure of the on-rate for binding, differences in off-rate may in part account for the above result. However, recent pharmacological studies indicate that the off-rates for ciguatoxins-1, -2 and -3 are similarly slow (Lewis & Wong Hoy,1993).

Ciguatoxins are sodium channel activator toxins that bind to site 5 on sodium channels, a site overlapping the brevetoxin binding site (Lombet et al.,1987; Lewis et al.,1991). Ciguatoxin binding leads to the opening of sodium channels which in turn results in an influx of sodium ions, cell depolarisation and the appearance of spontaneous actions potentials. The high affinity binding and subsequent alteration of voltage dependent sodium channels by ciguatoxins could form the basis of biosensor-type assays able to screen for the ciguatoxins. The technology for developing such biosensors is progressing rapidly (Ogert et al.,1992; Malmqvist,1993) but there are few commercial biosensor products available (Griffiths & Hall,1993). The advantage of biosensor technology is that the response can be proportional to level of sodium channel activator in a mixture. The mouse bioassay could be considered a crude form of biosensor. This assay has been validated for detection of ciguatoxins in up to 20mg of lipid extract from fish flesh (Lewis & Sellin, 1993). Cell based assays also show potential for detection of sodium channel blocking toxins (Gallacher & Birbeck,1992) and hold potential for detecting sodium channel activator toxins (Hungerford, 1993). Such assays also have the potential to be automated and miniaturised (Goguen & Kedersha,1993).

Ciguatoxin binding to other proteins

Ciguatoxins also have affinity for various proteins including IgG from a variety of sources and fish liver and fish flesh proteins (Parc et al., 1979; Emerson et al.,1983; Vernoux et al., 1985; Hahn & Capra,1992). The affinity of ciguatoxin for these proteins has not been quantified and these studies have failed to exclude the possibility that the binding they are measuring is not simply



CTX - 1 R_1 = OH

CTX - 2 R_1 = H

CTX - 3 R_1 = H

FIG. 2. Structures of the major ciguatoxins present in the flesh of carnivorous fish in the Pacific. CTX-2 is 52-epi CTX-3 (stereochemistry shown for CTX-1 and CTX-3).

the binding of ciguatoxin to the sodium channels present in the protein-containing extracts. Such an interaction is expected to be considerable in tissues rich in excitable cells (e.g. flesh). A putative ciguatoxin binding protein has been detected in fish flesh and its appearance has been proposed to result from the exposure of fish to ciguatoxin (Hahn & Capra,1992). Such a protein in fish may explain why fish are relatively less susceptible to the ciguatoxins than the closely related brevetoxins (Lewis,1992). An assay to detect such a protein in fish could provide the basis for a novel screening assay for ciguateric fish.

Any high affinity binding of ciguatoxins to proteins (including sodium channels) present in fish tissue could be used to immobilise ciguatoxin to a solid phase prior to detection with a labelled antibody specific for ciguatoxin-1. This approach can be considered a type of sandwich assay and could allow rapid detection of ciguatoxin in fish flesh without the need for a solvent extraction step. Such an approach warrants further investigation but requires an antibody that can 'see' the portion of ciguatoxin molecule not obscured as a result of its binding to the fish protein.

CHEMICAL FEATURES OF THE CIGUATOXINS

Structural features of the ciguatoxins

Ciguatoxins (Fig. 2) are lipid-soluble toxins consisting of 13 rings fused by ether linkages into a mostly rigid, ladder-like structure (Murata et al.,1990; Lewis et al.,1991,1993a); they are relatively inert molecules that can accumulate in fish to levels that are toxic to humans, a feature that distinguishes them from chemically and biochemically related brevetoxins which are lethal to fish before levels can be accumulated that cause human poisoning. Structurally related ciguatoxins-1, -2 and -3, are present in carnivorous fish (Lewis et al.,1991; Lewis & Sellin,1992; Lewis et al.,1993a). However, CTX-1 contributes most to the toxicity of ciguateric fish (Lewis & Sellin,1992) so an assay selective for CTX-1 would have general utility for detection of ciguateric fish. Involvement in human poisonings of other toxins (CTX-2, -3, gambiertoxins etc) in ciguateric fish remains unsubstantiated. The role in ciguatera for toxins other than site 5 sodium channel activator toxins is remote.

Extraction of ciguatoxins

Ciguateric fish contain 0.1–5ppb CTX-2 (Lewis & Sellin,1992; Lewis,1992). Whilst direct detection may be possible, extraction and clean-up procedures can be used to concentrate the ciguatoxins. The ciguatoxins are sufficiently hydrophobic to be insoluble in water but can be extracted from fish flesh with organic solvents such as methanol, chloroform or acetone. However, even with a several step clean-up procedure involving removal of low polarity lipids and water-soluble material, the ciguatoxins are still

present in the remaining lipid mixture at low levels of 30–5,000ppb. The lipid solubility and lack of a rapid, efficient clean-up procedure also have implications for antibody-based screening assays. Any organic solvents required to solubilise crude extracts containing ciguatoxins during incubation with an antibody may alter the three-dimensional structure of the antibody binding domain, thereby reducing the affinity of the antibody for ciguatoxin.

Chemical detection of the ciguatoxins

Pure CTX-1 can be detected to below 5ng by monitoring HPLC eluants with a sensitive u.v. detector (Lewis, unpubl. data). However, since the ciguatoxins do not possess a distinctive u.v. chromophore, it is not possible to develop a method for selectively detecting ciguatoxin in a crude lipid extract from fish by employing a u.v. detector to monitor the eluant from a HPLC run. As discussed previously, the major ciguatoxins found in fish each possess a relatively reactive primary hydroxyl through which labels could be attached to enhance detectability. Since numerous compounds in a lipid extract from fish might also react with such a label, detection of the labelled ciguatoxins must be done in combination with a subsequent separation technique. HPLC coupled to fluorescence detection provides a high sensitivity method that has the potential to detect natural levels of ciguatoxins in crude extracts from fish flesh. Such an analytical detection methodology could be used to validate positive (or negative) responses obtained by antibody-based assays. Dickey et al. (1992) reported encouraging results by labelling ciguatoxin with novel courmarin based fluorescent reagents sold by Molecular Probes Inc. (Eugene, OR, USA).

Although sensitive detection of anthroyl nitrile labelled ciguatoxin has been reported (Legrand et al.,1992), these approaches have not been extended to detection of ciguatoxins in crude extracts from fish. HPLC linked to a fluorescence detector was able to detect in relatively crude extracts ≥400ng diarrhetic shellfish toxins/g shellfish (Lee et al.,1987) and ≥13ng aflatoxin/g peanut butter or corn (Park et al.,1990). These approaches would require modification and significant improvements in sensitivity if the ciguatoxins in crude extracts from fish are to be detected. A simple clean-up procedure that minimises inference from lipids at both the labelling and detection steps is critical for the development of a sensitive test for ciguatoxins. HPLC coupled to selective-ion monitoring Ionspray mass spectrometry has shown considerable potential for the detection of labelled diarrhetic shellfish toxins (Pleasance et al,1992). Initial studies with CTX-1 indicate that such an approach could form the basis of a confirmatory analytical assay for ciguatoxins in fish (Dickey et al., in press; Lewis et al.,1993b). If sufficient unknown toxin is available (>25μg), NMR approaches can be utilised for confirmation or for characterisation of unknown toxins.

In addition to the major ciguatoxins isolated, low potency forms arising from further biotransformation of the ciguatoxins (Tosteson et al.,1988; Legrand et al.,1992; Lewis & Sellin, 1992; Lewis et al.,1992) may also accumulate in fishes. While low potency of these ciguatoxins makes detection by the mouse bioassay difficult, such compounds may cross-react with antibodies to CTX-1, thereby increasing the probability of obtaining false positive results. This problem arises because antibody-based assays respond depending on the relative affinity (specificity) of the antibody for each form of the toxin in a way that may only by chance be related to the potency of the different forms.

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